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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 8 1974

[redacted]

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐ Second Renewal ☒ Date: 1/28/74

1. Principal Investigator (give title and degrees):

Richard A. Lerner, M.D., Associate Member

2. Institution & address:

Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Department(s) where research will be done or collaboration provided:

Department of Experimental Pathology

4. Short title of study:

Studies on Persistent Viral Infection

5. Proposed renewal date: July 1, 1974

6. How results to date have changed earlier specific research aims:

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7. How results to date have changed earlier working hypothesis:

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Source: https://www.industrydocuments.ucsf.edu/docs/jfam0000

8. Any additional facilities now required? Describe briefly:

no additional facilities required

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Addition of Chi-Hung Siu

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

See attached list

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12. Summary progress report (append in standard form as separate document, unless recently submitted).

Source: https://www.industrydocuments.ucsf.edu/docs/jldm0000

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Richard A. Lerner	50%	-0-
Frank J. Dixon, Consultant	--	-0-
David Kohne	100%	-0-
Fred Jensen	100%	-0-
Alvaro Puga	100%	10,480
Stephen J. Kennel	50%	-0-
Bert C. Del Villano	100%	-0-
Chi-Hung Siu	100%	8,000
Technical		
Catherine Morris, Technician	100%	9,000
Part-time services of: secretary, animal caretakers, photographer, glass washers, histology technician, EM technician, machinist & electronic repairman		7,200
	Sub-Total for A	34,680

B. Consumable supplies (by major categories)	
Tissue culture media, glassware and plasticware	8,000
Isotope labeled compounds	5,000
Liquid scintillators and vials	5,000
Sub-Total for B	18,000

C. Other expenses (itemize)	
Photography, histology, part-time use of electron microscope	4,170
Sub-Total for C	4,170
Running Total of A + B + C	56,850

D. Permanent equipment (itemize)

None

Sub-Total for D	-0-
E. Indirect costs (15% of A+B+C)	8,528
Total request	65,378

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Source: https://www.industrydocuments.ucsf.edu/docs/jdm0000

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Studies on persistent viral infection	Council for Tobacco Research #766A	$47,385	7/1/73-6/30/74
Molecular structure of the immunoglobulin receptor in continuous cultures of diploid human lymphocytes	National Science Foundation GB 34296	$65,000	6/1/72-11/30/74
Immunopathology of virus infection (Salary - RAL)	NIH Career Development Award AI-46372	approx. $125,000	7/1/70-6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Richard A. Lerner, M.D.

Signature Richard A. Lerner Date

Telephone 714/459-2390, ext. 470
Area Code Number Extension

Checks payable to

Scripps Clinic and Research Foundation

Mailing address for checks:

Mr. O.K. Kincaid, Jr., Controller
Scripps Clinic and Research Foundation
476 Prospect St., La Jolla, Calif. 92037

Responsible officer of institution

Typed Name Edmund L. Keeney, M.D.

Title President and Director

Signature Edmund L. Keeney Date 2/1/74

Telephone 714/459-2390, ext. 367
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10. Outline of Experimental Protocol

A. Introduction

This project continues to offer a multidisciplinary study of oncornaviruses, and the nature of their interaction with their host which may lead to the formation of tumors or to the development of immunologic diseases or both. The disciplines involved are virology, molecular biology, biochemistry and immunopathology. The immunologic emphasis in this work relating both to the immunopathologic consequences of oncornavirus infection and to the use of immunologic markers of viral presence are perhaps a unique aspect of this proposal. A better understanding of the immunologic consequences of oncornavirus infection is needed if we are to understand viral oncogenesis and host defense mechanisms, and to achieve effective therapeutic and/or phrophylactic immunotherapy and also to avoid the possible immunopathologic complications of such therapy. The studies proposed are designed to contribute toward such an improved understanding.

B. Objectives and Scope

This project focuses on several interrelated aspects of the oncogenic and immunopathologic consequences of oncornavirus infection with particular emphasis on the situation in NZ mice. The first of these is the possible role of SLV, an oncornavirus(es) obtained from NZB lymphoblast cultures, in the genesis of spontaneous tumors and immunologic diseases of the NZ mice. Second, using SLV as a model oncornavirus, we are attempting to find if and how in vivo infection of a variety of murine and rat strains with this agent will cause tumors and immunologic diseases. Third, we plan to isolate and characterize the glycoprotein surface antigens of oncornaviruses and use this antigen as one of the yardsticks in evaluating the host-virus relationship. Fourth, we are analyzing the number and nature of the viruses produced by continuous thymoblast lines derived from NZB and related mice. Fifth, we propose to correlate a variety of virion coded or induced phenotypic markers of infected lymphoblasts with their oncogenic or immunologic potential in vivo. Sixth, we will study the incorporation of exogenous nucleic acids into oncorna and other RNA viruses and attempt to determine whether this process may play a role in the induction of "autoimmune" responses following infection with their viruses. And seventh, we are searching for human C type viruses in the placentas of humans with lupus erythematosus.

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The concept of immunological tolerance to viruses causing persistent infection after intrauterine or neonatal infection was first challenged seven years ago by the demonstration that mice vertically infected with LCM did in fact make an immune response to the virus and that this response caused a fatal immunologic disease (Oldstone and Dixon, Science, 158:1193, 1967; Oldstone and Dixon, J. Exp. Med. 129:483, 1969). Since then numerous similar reports have identified immune responses to many persistent viruses including the oncornaviruses (Oldstone, Aoki and Dixon, PNAS, 69:134, 1972) with associated immunological diseases of varying severity. The manifestations of these immune responses, i.e., antibodies and sensitized cells, can serve as indicators of the presence of such infections and in the case of oncornaviruses provides one of the best and at times the only evidence

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of their existence. The fact that antibody responses to oncornaviruses may be associated with particular immunologic diseases such as immune complex nephritis, or more specifically, lupus type nephritis, prompts a search for oncornaviruses in patients with these conditions. Several laboratories including our own have identified the initiation of antinuclear antibody (ANA) formation in animals as a result of oncornavirus infection further linking these viruses to human immunologic disease and suggesting that nuclear antigens may be presented in a particularly immunogenic form as a result of oncornavirus infection. Thus, the pathologic package offered by oncornavirus infection may include tumors for the least immunologically responsive host and immunologic disease for the most responsive with appropriate combinations for those in between. The experiments described below are designed to determine whether this hypothesis is true and if so how the virus acts to initiate either tumors or self destructive immune responses and what factors in the host determine the pathological outcome of such an infection.

C. Background (see Progress Report)

D. Experimental Procedures

1. Effects of neonatal infection of mice and rats with SLV. We intend to complete our ongoing study of the oncogenic and immunopathologic consequences of neonatal infection of mice and rats with SLV as described in the accompanying progress report. Our purpose of this initial screening approach is to identify strains of mice or rats which react oncogenically or immunopathologically in particular ways to SLV infection, thus providing us with host-virus combinations which may be studied for the particular factors predisposing to specific disease states. Although this study has been underway for only a few months, we already have several promising leads which appear worthy of follow-up.

a. Factors regulating serum gs levels in animals infected as neonates with SLV. The 2-3 month serum gs levels of all strains of SLV infected mice and rats are 10 to 60 times greater than normal, except for the NZB x W and NZW x B mice in which gs levels are not significantly elevated. The low serum gs levels in the NZ hybrid mice could be the result of either the failure of SLV to infect or multiply in these animals or the operation of a suppressive immunologic response which was effective in rapidly removing the gs and perhaps other viral antigens from the circulation. In order to test these hypotheses, we will conduct a combination of in vivo and in vitro studies. The tissues (spleens, thymus, lungs and kidneys) of SLV infected NZ hybrid mice taken at 1, 3 and 6 months of age will be assayed by quantitative immunochemical procedures for gs and GP-70 SLV antigens in order to determine whether these viral products are being produced in the tissues. These results from the NZ hybrids will be compared with results of similar observations made in the high serum gs strains, thereby providing some idea of relative rates of oncornavirus synthesis in the different strains. In vitro studies will include

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the growth of primary cultures of spleen, thymus, lungs and kidneys from SLV infected NZ hybrids and from SLV infected high serum gs strains and the determination of the amount of infectious virus formed. These two procedures should indicate whether SLV can and does infect and replicate in the NZ hybrids as well as in other strains. If it can the results would suggest that the low serum gs levels in the former are the result of immune elimination of the viral antigens by circulating antibody and this would fit with the well recognized immune complex disease from which these mice suffer.

b. The basis for resistance of NZ hybrids and C57 Black/6J mice to SLV induced tumors. All the strains of mice and rats infected with SLV in our screening study except the NZ hybrids and to a lesser extent the C57 Bl/6J develop a high incidence of lymphoma and/or leukemia. The means by which these two kinds of mice respond to and control the oncogenic effects of SLV infection are probably different in view of the widely differing gs levels they have. In order to get some idea of the virus-host relationship in these resistant strains we will check them at 1, 3 and 6 months for neutralizing antibody to SLV and also see whether the virus present in each has the same biological behavior. To determine the latter we will isolate virus from SLV infected NZ hybrids, C57 Bl/6J and from one or two high leukemia strains and titrate these isolates by X-C test in vitro and inject them into neonatal Balb/c mice to determine their leukemogenicity in vivo. These two lines of study should give some idea of the nature of the resistance of these mice, i.e., whether they are resisting an oncogenic virus or whether the virus replicating in them is not oncogenic.

c. The nature of ANA formed by mice responding in different ways to SLV infection. High levels of ANA are observed in two groups of mice responding quite differently to SLV infection. In the NZ hybrid strains there is considerable ANA production and a high incidence of severe glomerulonephritis, low incidence of lymphoma and low levels of circulating gs. In the C57 Black/6J and C57 Black/10J there is a high level of ANA with little or no glomerulonephritis, a low to moderate incidence of lymphoma but high circulating gs levels. The immunologic specificity of the ANAs made by these two groups of animals will be compared by quantitative and qualitative immunochemical procedures. The spectrum of antinuclear antibodies in each of these types of sera will be determined by measuring the amount of antibody capable of reacting with double stranded DNA, with single stranded DNA, with double stranded RNA and single stranded RNA by modifications of the Farr technique which are currently employed for these determinations in our laboratory. In addition by absorption and blocking procedures using both antigen binding tests and fluorescent antibody determinations as end points we can determine the approximate amount of antibodies in these sera against histones, nucleo-protein, and the SM nuclear antigen. These studies should provide valuable leads as to the immunopathogenicity of various ANAs. They may also allow us to learn whether the immunogenic challenges presented by SLV in the different strains of mice are different or whether the genetic backgrounds of the mice dictate different responses to similar antigenic challenges.

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d. The immunologic basis for the glomerulonephritis of SLV infection associated with high and low ANA responses. A high incidence of glomerulonephritis was observed in both the New Zealand hybrid strains associated with a high ANA response and in the C57 brown mice with a low ANA response. Since the pathogenetic mechanism in this form of glomerulonephritis is almost certainly antibody mediated we plan to compare the antibodies found in the glomeruli of the diseased kidneys of these two kinds of mice. The kidneys from approximately 30 to 40 nephritic mice of each kind will be pooled, homogenized, washed repeatedly in cold saline and then the residual immunoglobulin eluted at pH 3.2 in citrate buffer (J. Exp. Med. 127:507, 1968). This treatment removes the Ig bound immunologically in the kidney and allows one to determine the specificity of the antibodies involved in the nephritogenic process. The eluates will be neutralized and concentrated to a convenient volume. The concentration of Ig in the eluates can then be determined by radioimmunodiffusion and aliquots of the eluates can be absorbed by various insoluble nuclear and viral antigens after which the Ig concentration is again determined and from the difference in the pre and post absorption concentrations, the percent of immunoglobulin eluted which is reactive with the antigens employed in absorption can be obtained. With the particular strains selected by our screening procedure one might expect to find quite different antigen-antibody systems involved in the nephritogenic process in the respective strains. Such observations would help define the nature of nephritogenic immune complexes induced by oncogenic viral infections and by the nature of the antigens involved might tell us a good bit about the role of viral infection in antigen liberation. These observations would also go a long way toward determining whether the immunologic renal disease produced by infection with these viruses is antiviral in nature or "auto" immune.

2. Influence of immunosuppression and immunotherapy on the course of spontaneous and induced SLV infection and oncogenesis.

Of all the strains of mice observed in our screen to date, only the NZ hybrids and to a lesser degree the C57 Black/6J mice appear highly resistant to the development of lymphoma. We propose to try to ascertain the importance of the immune response in these animals in the resistance to lymphoma. Both the New Zealand hybrids and the C57 Black/6 mice will be infected with SLV at birth as in the screening study and then as soon as possible put on continuing immunosuppressive doses of Imuran or comparable agents. NZ hybrid and C57 Black/6 mice uninoculated with SLV will be similarly subjected to immunosuppression and serve as controls. These animals will be followed for the development of tumors, glomerulonephritis and ANA. The conduct of these experiments will be similar to that for the screening experiment now underway (see Progress Report). The degree of suppression of the cellular and humoral immunologic processes will be tested by appropriate antigenic challenge during the course of the experiment. If as seems likely we are able to modify the course of SLV infection by this kind of immunosuppression and for example observe a high incidence of lymphoma, it would suggest that the immune response or something else affected by Imuran

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was playing a significant role in the repression of tumors in the unsuppressed animals. In addition, if we observe tumors, particularly lymphomas, in the immunosuppressed but uninfected mice of either strain it would suggest that these animals do carry potentially oncogenic viruses which are held in check apparently at least in part by immunologic mechanisms.

If such an immunosuppressive regime were effective in inducing tumors in SLV infected animals otherwise resistant to oncogenesis, the suppressed animals might provide a useful model for immunologic manipulation of developing tumors. Termination of immunosuppression, with or without passive transfer of antibodies or syngenic sensitized cells, could be used in attempts to control the growth of a "spontaneous" tumor. In such a therapeutic situation the course of ancillary immune responses such as ANA and the diseases such as glomerulonephritis associated with them could also be observed to see whether such an untoward immunopathologic development would occur in the course of experimental immunotherapy.

Immunotherapy of viral oncogenesis can also be attempted in mice such as Balb/c and SWR/J which have a high incidence of lymphoma after neonatal SLV infection. Since adult mice of these strains are resistant to the oncogenic effects of either SLV infection or transfer of malignant SLV producing lymphoblasts, transfer of syngeneic adult lymphoid cells to neonatally infected mice can be attempted at various times before and after appearance of the tumor in order to observe the efficacy of such immunotherapy and the possible development of immunopathologic complications.

3. The effect of neonatal oncornavirus infection on the immunologic properties of mice. Since oncornavirus infection produces a number of immunologic responses in non-New Zealand mice which are characteristic of the immunologic disease picture seen in unmanipulated New Zealand mice, and since it has been claimed that there is a deficiency in the cellular immune mechanisms and an associated hyperresponsiveness in the humoral immune mechanisms of unmanipulated New Zealand mice, it would be interesting to determine whether the oncornavirus infection in non-New Zealand mice produce changes in the immunologic system making them similar to New Zealand mice. To do this we plan to infect neonatal Balb/c x NZB hybrids, C57 Brown and C57 Black/6 mice with SLV. Their cellular and humoral immunologic responsiveness will be tested periodically by attempted induction of immunologic tolerance, by in vitro responsiveness to sheep red blood cells using the Jerne plaque technique, and by the humoral and cellular responses to various antigens as determined by primary antigen binding tests and by lymphocyte transformation or MIF production. If oncornaviruses such as SLV and Moloney which do induce autoimmune type responses in mice also produce the same changes in the immune system that are found spontaneously in New Zealand animals, it would suggest that the primary effect of these viruses on the lymphoid system is important in producing the aberrant responses. If on the other hand, the viral infections do not produce such a functional change in the lymphoid system, it would suggest that these viral infections operate via the release and/or abnormal presentation of certain endo-

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genous and/or exogenous antigens. While there have been a number of reports of alterations in the immune responses of animals infected with oncogenic viruses, there has not been a study carried out designed to show whether these immunologic alterations might be important in the development of immunologic disease.

4. Isolation and characterization of the glycoprotein surface antigens of oncornaviruses and leukemic cells. Virus induced cell surface antigens of leukemia cells are of importance as they represent targets for the immune defense of the host. These antigens may represent markers indicating the degree of expression of an oncogenic viral genome and they may also affect cell membranes in a way which leads to the altered behavior of transformed cells. A review of the extensive serological studies of Gross virus antigens has been presented by Aoki et al. (Aoki, T. et al., J. Virol. 10:1208, 1972). Although these approaches have led to advances in the study of tumor antigens, they have certain limitations, as data from these serologic techniques bear no direct relation to antibody interaction with specific membrane components. In order to gain a better understanding of the complex role of viral envelope antigens in oncogenesis, it is necessary to isolate them and to determine their immunochemical and molecular characteristics.

a. Objectives and scope. Our major aims are 1) to isolate sufficient quantities of viral envelope antigens required for their thorough immunochemical and chemical characterization and 2) to utilize these well defined reagents to produce antibody suitable to assess the biological relevance of these viral antigens for the overall problem of carcinogenesis. Specifically, we will assess the role of these antigens in viral infectivity by determining their capacity to produce specific neutralizing antibody. We will also attempt to evaluate to what extent antigenic determinants are shared between viral envelope antigens which differ widely in their tropisms as we utilize two B- and two N-tropic viruses, respectively. Ultimately, it will be of key importance to determine whether reagents developed during this study can be utilized to inhibit tumor growth in the animal model system.

b. Background. Soluble hemagglutinating surface antigens were isolated by enzyme treatment of several murine leukemia viruses (Whitter, R. et al., Virology 54:330, 1973). These antigens were able to inhibit potent neutralizing antisera directed against tween ether degraded virus particles. Removal of these surface antigens by bromelin treatment resulted in complete loss of virus infectivity. These antigens were shown to have a molecular weight of 93,000 and appeared to be glycoprotein in nature. The isolation of Rauscher murine leukemia virus polypeptides containing an interspecies antigen was recently reported by Strand and August (Strand M. and August, J.T., J. Biol. Chem. 248:5627, 1973.) These investigators utilized freezing and thawing together with sonication to solubilize these antigens which upon further purification by ion-exchange chromatography yielded two components upon SDS-acrylamide gel electrophoresis with molecular sizes of 69 and 71,000, respectively (GP 69-71). Radioimmuno-

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assay with anti-feline virus serum revealed that both polypeptides contained the interspecies determinants. It is believed that these antigens are virus coded due to the selective nature of the interspecies antigenic determinants and their absence in uninfected cells.

A glycoprotein of 70,000 molecular weight (OSA) was detected on an oncogenic C-type virus, Scripps leukemia virus (SLV) produced by a lymphoblastoid cell line from an NZB mouse (Kennel, S. J. et al., Virology 55:464, 1973). By selective radiolabeling, OSA was shown to comprise 0.1% of total cellular amino acids and 10% of the cellular glucosamine. This protein was found to react with sera which neutralized Moloney, Kisten, Rauscher, AKR and Scripps viruses and is believed to be the antigen which may be involved in virus neutralization.

c. Experimental procedure--production of antibody against SLV GP 69-71. To be able to develop a meaningful radioimmunoassay for SLV GP 69-71, we will utilize the purified antigen on hand to produce specific antibodies in rabbits. For this purpose we will make bi-weekly injections in complete Freund's adjuvant (CFA) initially by injecting the material in the lymph nodes to be followed by bi-weekly subcutaneous injections in CFA. We will ascertain whether any neutralizing antibody is thus produced by testing the rabbit sera in the XC-cell plaque assay as described by Rowe et al. (Rowe, W. P., Virology 42:1136, 1970).

Development of radioimmunoassay for SLV GP 69-71. We will utilize the SLV GP 69-71 isolated thus far to develop a radioimmunoassay essentially as described by Hunter (Hunter, W. M., in Handbook of Exptl. Immunology, Weir, P. M. ed., pp. 608-642, 1967, F. A. Davis, Philadelphia). This assay will permit the quantitative analysis of viral antigens and will be utilized to measure the progress of viral antigen isolation, purification and chemical characterization. The antigenic profile of GP 69-71 antigens isolated from SLV, RLV, wild mouse and AKR leukemia viruses will be compared by use of the competitive radioimmunoassay. To determine whether GP 69-71 is not just a cell membrane component, it will be necessary to test whether either uninfected host cells or those infected with another membrane maturing virus, e.g., VSV, also contain GP 69-71.

Assessment of GP 69-71 on wild mouse (1504E) and AKR virus. To determine whether these two viruses contain GP 69-71, we will first assess whether neutralizing antibody produced against SLV GP 69-71 can be inhibited by these two viruses. Once we thus establish the presence of GP 69-71 on wild mouse and AKR viruses, we will attempt to isolate these antigens essentially by the same methods developed by us for the isolation of SLV GP 69-71.

Antigenic comparison of GP 69-71 of different viruses. We will determine whether envelope antigen from SLV, RLV, AKR and wild mouse viruses (1504E) share common antigenic determinants. For this purpose we will absorb neutralizing

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antisera directed against purified GP 69-71 of these above viruses with cultured NZB fibrosarcoma cells which are essentially free of SLV but possess GP 69-71 antigens. The residual neutralizing capacity of these absorbed antisera against intact viruses will then be determined. We will also utilize competition radioimmunoassays and gel diffusion analyses to compare antigenic profiles of the GP 69-71 components of different viruses.

Chemical characterization of SLV GP 69-71. We will first evaluate the degree of charge homogeneity of the isolated antigens. To this end we will individually cut out these components from SDS - acrylamide gels, remove the SDS by extensive dialysis against 10M urea, and analyze the antigen bands on urea-acrylamide gels for charge heterogeneity under different conditions of pH and pore size.

If there exists any charge heterogeneity we will determine whether this is due to either carbohydrate, i.e., sialic acid and/or amino acid composition. We will thus treat GP 69 and GP 71 components with neuraminidase and then evaluate their electrophoretic profiles in urea-acrylamide gels.

To ascertain whether irrespective of any charge differences, GP 69 and GP 71 contain a different peptide composition, we will analyze them by tryptic peptide maps. To this end we will utilize similar techniques as those used by Moss (Moss, B. et al., Virology 55:143, 1973). Thus, we will label the NZB cultured cells in situ with a mixture of ^{14}C amino acids or with ^{14}C glucosamine and isolate GP 69 and GP 71 from semi-preparative (1.6 mm) acrylamide gels by cutting out the two bands which contain these antigens. The proteins will be eluted with SDS, precipitated in TCA, dissolved in performic acid, lyophilized and then digested with trypsin. Peptide maps will be made on cellulose thin layer sheets and the patterns visualized with x-ray film. This approach should make it possible to evaluate to some extent the structural differences in these two molecular size classes of viral surface antigens. Labeling with ^{14}C hexosamine will tell us whether or not structural differences between the two moieties are located in their glycopeptide components. We will also determine the amino acid composition of SLV GP 69-71 and of the individual GP 69 and GP 71 components, respectively. A special effort will be made to carry out careful performic acid oxidations to ascertain the number of cysteic acid residues to give us an idea of the disulfide bridge content of these molecules. If disulfide bridges are present, we will completely reduce and carboxamidomethylate the components and then assess by acrylamide gel electrophoresis in the presence of either SDS or urea whether the antigenic moieties consist of one or more polypeptide chains.

An effort will be made to establish the carbohydrate profile of SLV GP 69-71. For this purpose we will utilize gas chromatographic analyses already ongoing in our laboratory. Depending on carbohydrate composition and sialic acid content, we will utilize either neuraminidase or specific purified glycosidases (on hand in our laboratory) to digest these antigens while monitoring their remaining carbohydrate residues by gas chromatography. This approach

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should allow us to determine the role which carbohydrate moieties play in expressing the antigenicity of GP 69-71. In particular, it would be of interest if there would be any change in the inhibitory capacity of these antigens in virus neutralization assays following the removal of carbohydrate groups or whether antigens treated in this manner vary greatly in their reactivity in the radioimmunoassay.

Finally, we will attempt to determine the minimal molecular size required for GP 69-71 to express its antigenic activity. To this end we will use controlled proteolysis with insolubilized trypsin at non-optimal pH for digestion to obtain relatively large protein fragments. Such moieties could then be tested in the radioimmunoassay or assayed for their capacity to inhibit neutralizing antibody.

5. Studies on the number and nature of viruses produced in continuously growing thymocyte lines established from the New Zealand mice. From the work cited above, as well as the studies of others, it is clear that oncornaviruses may play a role in the pathogenesis and etiology of the autoimmune disease of the New Zealand mice. However, since all of the studies, including our own, have been carried out with a complex mixture of oncornaviruses, it is of extreme importance to characterize in as much detail as possible the nature of the viruses responsible for the various pathological processes observed when they are injected into newborn mice.

a) A single virus could cause more than one disease such as tumors and autoimmunity in a single mouse.

b) A single virus might cause one disease in one strain of mice and a different disease in another strain of mice.

c) Each of the different viruses in the mixture could be responsible for a specific and different pathologic manifestation upon injection into newborn mice.

In order to begin to segregate these possibilities, we are studying the nature of the viruses present in the supernatant fluid harvested from our continuous thymocyte cultures. During the past year, we have determined that the supernatant fluid of continuously growing thymocytes derived from the New Zealand Black mice contain not only a murine C-type oncornavirus but also a particulate fraction with a density of 1.19 gm x cm^{-3}. This fraction is composed of viral C type and B type (MMTV) particles as well as cellular membranes and organelles. An RNA dependent DNA polymerase (RDDP) as well as a DNA dependent DNA polymerase are present in this fraction, which also contains an endogenous template for the synthesis of DNA. The RNA found in the 1.19 gm x cm^{-3} band does not have a 60-70S sedimentation coefficient which is characteristic of the

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C-type and B-type viruses, but instead sediments at 20-30S. DNA of murine origin is also found in association with the viral core particles which were made by treatment with NP-40. Serial passages of the supernatant fluid from 60A lymphoblast cultures (containing a mixture of C-type virus and particles from the 1.19 gm x cm^{-3} density class) on secondary BALB/c fibroblasts gradually resulted in a loss of the particles with a density of 1.19 gm x cm^{-3}, and by the sixth passage none of these particles were observed. Concomitantly, preliminary evidence indicated that the virus progeny obtained from these passages on fibroblasts lost its leukemogenicity, as determined by injection into newborn mice. Because of these results, several questions have become pertinent:

a) We would like to know what the relationship is between the viral genome and the nucleic acids (DNA and RNA) found in the 1.19 gm x cm^{-3} band.

b) If the long term animal inoculations corroborate the preliminary evidence that leukemogenicity is lost when the virus is passed on fibroblasts, it will be important for us to determine what correlation can be made between the nucleic acid sequences found in the 1.19 gm x cm^{-3} band and the biological activity of the viruses.

Experiments to answer some of these questions are already in progress. DNA "probes" will be made using the DNA polymerase and template endogenous to either the viral (1.16 gm x cm^{-3} density class) preparation or to the 1.19 gm x cm^{-3} density class. DNA "probes" will also be made from the virus isolated from the milk of lactating NZB mothers. These "probes" will be hybridized to viral RNA, the RNA and DNA found in the 1.19 gm x cm^{-3} band, to uninfected (whole BALB/c embryo) cell DNA and to DNA extracted from 60A cells. The hybrids will be detected by chromatography on hydroxyapatite and the fraction of the DNA "probe" that hybridizes in the homologous versus the heterologous system will give an indication of the relatedness of the nucleic acids under study.

In order to place all of these studies relative to oncorna viruses and the etiology of autoimmunity into proper perspective, it is necessary to determine which of the many "candidate" viruses are endogenous to one or another colony of New Zealand mice, and if there is any single oncorna virus which seems to be present in all strains of NZ mice. Probably the best way to determine this is to:

a) determine the genotypic taxonomy of the various viruses in question, and

b) attempt to isolate in separate laboratories viruses from several colonies of New Zealand mice.

In order to carry out the experiments indicated in a), it is necessary to

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have representative DNA "probes". Experiments in our laboratory as well as in those of Dr. Raymond Gilden and Dr. David Baltimore indicate that the genome of SLV is closely related to that of Moloney virus. These experiments have involved the preparation of DNA "probes" from both viruses using RDDP from and the endogenous viral template. Hybridization of RNA extracted from SLV and Moloney to the "probes" was studied. A small difference (2-6%) was observed. None of the laboratories cited have used DNA "probes" with a balanced representation of the viral genome. The "probes" which have been used are total RDDP products, which have been selected by hybridization to a large excess of viral RNA. These "probes" have been shown to be unbalanced with certain viral sequences being represented 10-24 fold more frequently than others. Thus, it is not known whether the small differences observed are the result of lack of representation or correspond truly to a difference between the genomes of the two viruses. We now have the capability for preparation of representative DNA "probes" from the SLV genome by hybridization of the "probe" in DNA excess to radioactively labeled viral RNA. Determination of the extent to which the RNA is protected from digestion by RNase A in high salt is carried out to insure that the RNA is nearly completely resistant to digestion and thus the "probe" is a completely representative complement to the RNA. Digestion of the unhybridized DNA sequences with the endonuclease S_1 or hydroxyapatite chromatography will render a representative DNA copy of the viral genome. If using this DNA "probe" means that the small differences between the genomes of the two viruses still hold true, we will be able to amplify the signal by recycling the unhybridized "probe" and asking the question whether the 2-6% of the SLV "probe" that does not hybridize to Moloney RNA can hybridize 100% to SLV RNA and vice versa. With the recent interest in the xenotrophic viruses, a similar experimental approach can be taken to determine the relationship of the xenotrophic genome to Moloney and/or SLV viruses. Toward this end, we intend to exchange reagents and "probes" with Dr. Raymond Gilden. To accomplish the objective indicated in b), New Zealand mice have been obtained from Stanford University, Columbia University and we are expecting New Zealand Black mice from Otago University, New Zealand. We will establish lymphoblast cell lines from these mice and those which produce virus will be studied for the relationship of their virus to SLV. In addition, we are studying viral isolates from the NZW and (NZB x NZW)F_1 hybrid mice. In order to rule out any possibility that we are dealing with a contaminant which originates from a single lab, a collaborative effort will again be undertaken with Dr. Raymond Gilden in which mice are exchanged and lymphoblast lines are established in both laboratories.

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6. Possible role of incorporation of exogenous nucleic acids into oncorna and other RNA viruses in induction of autoimmunity. One of the basic reasons for suspecting that oncornaviruses may play a role in the etiology of the autoimmunity of the New Zealand mice and perhaps systemic lupus erythematosus in the human is that both these mice and patients with SLE make antibody not only to a single nucleic acid but to both DNA and RNA macromolecules as well as other nuclear and nucleolar macromolecules. Thus, in terms of classical immunology, one was tempted to

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search for an agent which during its natural history exposes the host to as wide a spectrum of potential nucleic acid immunogens as possible. Because of the fact that oncorna virus RNA is transcribed into DNA, and thus a variety of nucleic acid intermediates are involved, they are attractive candidates. Another intriguing possibility is that any virus which might contain both DNA and RNA could be a potential immunogen in autoimmunity. Again, because of the reports of Bishop et al., Smith et al. and Puga et al. who showed that the avian oncorna viruses, A type particles, and murine oncorna viruses contain DNA as well as RNA, the possibility that oncorna viruses immunize because of fortuitous packaging of both DNA and RNA must be considered. More recently, we have shown that "ordinary" RNA viruses can be made to incorporate cellular DNA. The New Jersey strain of vesicular stomatitis virus, when grown in suspension cultures of diploid human lymphoblasts, packaged host DNA which presumably originates from the plasma membrane associated DNA which we have shown to be present in these cells. This DNA is a linear duplex with a molecular size of approximately 900,000 daltons and by reassociation kinetics can be shown to be of host origin and not to be derived from contamination with viral, mycoplasmic or mitochondrial DNA. Because of the very wide host range, and somatotrophism of vesicular stomatitis virus, the potential for exchange of genetic information amongst species or different cells within a species is a real possibility. Our initial experiments with the DNA present in the oncornaviruses as well as that present in vesicular stomatitis virus will be aimed at determining the exact relatedness of the DNA in the virion to the host genome. In the oncorna virus system, radioactive DNA precursors will be added to 60A cells and purified 1.19 gm x cm^{-3} particles will be prepared. In order to avoid inherent problems due to possible contamination with random DNA fragments, these particles will be treated with DNase and cores will be made by treatment of the particles with non-ionic detergents and sedimentation either in sucrose or CsCl gradients. Only that DNA which is protected from digestion with DNase and sediments with the ribonucleoprotein cores will be used as a "probe". This DNA will be hybridized to viral RNA, to the RNA and DNA found in the 1.19 gm x cm^{-3} band, and to uninfected (whole BALB/c embryo) cell DNA and to the DNA extracted from 60A cells. By this means, we will be able to determine the origin of the DNA present in the oncorna viruses as well as its relationship to various subfractions of the host genome. It is most likely that this DNA will be similar to that already studied in the avian system and will be representative of the bulk of the murine genome. Similar experiments have already been carried out on the DNA isolated from the cores of vesicular stomatitis virus. Since, as yet, this DNA has not been obtained in large quantities,only limited studies relative to the analytical complexity of this DNA have been performed. Nevertheless, these studies are of extreme interest, in that they indicate that only unique sequences of the lymphocyte genome are packaged into the VSV cores. During the next year, we intend to study in great detail the analytical complexity of the DNA contained in the vesicular stomatitis virus cores to determine its exact relationship to the human genome. One intriguing aspect of all this is that there is a rearrangement of sequence relatedness and complexity relative to the host genome. For example, even though a sequence may be repeated many,many times in the host genome, if only one copy is present in the virions derived from those cells, then as far as

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the virion mass is concerned, this sequence is unique. Once we determine the analytical complexity of the DNA present in the VSV cores, we intend to turn to the question of whether vesicular stomatitis virus can pick up "oncogenes" and deliver them in a meaningful fashion to cells from different species and/or different somatic types. In many respects, VSV is an ideal virus for such studies. As mentioned above, it has a wide host range and somatotrophism. Perhaps more important, because it contains a large number of defective interfering particles which do not kill host cells, the potential for a meaningful exchange of genetic information is enhanced. We plan to infect with VSV malignant cells which are not producing RNA or infectious virus. After infection with VSV, the T or defective interfering particles will be extensively purified and their oncogenic potential will be studied in both in vitro and in vivo systems. If these particles can be shown to be oncogenic, then a very important system for encapsulation of "oncogenes" may be realized. In addition, with respect to their autoimmunogenic potential, we will immunize animals with similar particles and determine whether they can induce autoimmunity in susceptible strains. Again, one reason to suspect that this may work is that such particles may induce cells to replicate and synthesize nucleic acids foreign to the host even in the absence of production of complete virus.

7. Relationship of virion coded for and/or induced phenotypic markers to oncogenesis and autoimmunity. Cells transformed by oncorna viruses are characterized by a number of "markers" such as: 1) production of complete virus particles (electron microscopy), 2) production of infectious virus particles (XC), 3) presence of RNA dependent DNA polymerase, 4) presence of GP70 on the cell surface (interspec 2, OSA), 5) presence of P30 (gs-1), 6) oncogenicity.

Continuous lymphoblast cultures from NZB mice (SCRF 60A) have all of these "markers". To determine the relationship between each of these markers and the transformed phenotypes, SCRF 60A will be cloned in microculture plates. Replicate cultures can then be obtained using a replicate plating device designed by Dr. Jim Robb. The master culture can then be frozen using dimethylsulfoxide until variant cells can be characterized. With antisera prepared in our lab, we can then select by cytotoxicity and screen by immunofluorescence literally thousands of clones to obtain cells with phenotypes such as OSA gs+, OSA+XC-, etc. Certain variant cells can then be grown to mass culture and tested for their oncogenic potential in syngeneic adult and newborn mice. These variant cells will allow us to determine which of the virus coded and/or induced phenotypes are necessary for oncogenesis and which, if any, confer negative selective pressure for tumor growth. For example, from the data which we already have from our study of spontaneously occurring solid tumors, one might expect OSA-gs+ or OSA-rt+ cells to be highly oncogenic.

The virus produced by SCRF 60A cells (SLV 60A) has been shown to be a mixture of particles, some of which have a defective genome and/or contain cellular DNA. SLV 60A can be used to infect mouse or rat embryo fibroblasts in microculture plates. The techniques described above can then be applied to these cultures to clone viruses with defective genomes. In fact, this technique

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could be used to isolate temperature sensitive mutants of SLV. By this approach, we may be able to further elucidate the role of the various viral functions in oncogenesis and autoimmunity. To establish this end, cellular variants or defective viruses will be injected into newborn mice and the mice studied for the development of tumors and autoimmune disease. As far as autoimmunity is concerned, it will be interesting if the cell which has a phenotype such as V^-, OSA^-, rH^+, gs- could, because of production of a potential array of nucleic acid immunogens, induce autoimmunity. It, of course, remains to be determined if cells or viruses with the exact phenotypes which we would like to have can be obtained, but this seems to us to be an important approach to the problem of the relationship between autoimmunity and neoplasia.

In addition, DNA-DNA reassociation kinetics will be utilized to compare the number and completeness of SLV genome equivalents present in the variant cell genome to that of virus infected cells so as to determine whether a complete gene complement is present. In this way, we should be able to discriminate between the absence of a complete genome and the failure to express certain genes. To do this we will utilize DNA "probes" which were prepared last year. In the preparation of these "probes", we were careful to select DNA molecules which are most representative of the complete viral genome. We expect this work to complement our ongoing effort which has already shown that, at least as far as the New Zealand mice are concerned, the spontaneously occurring solid tumors rarely, if ever, express all the markers indicated above.

8. Search for C-type virus in humans .

Recently it has been shown that C-type viruses are present in the syncytial trophoblasts of primates and humans. Because of the possible role of these viruses in the etiology and pathogenesis of systemic lupus erythematosus (SLE), we are making attempts to isolate and characterize a C-type virus from the placentas of patients with SLE. To date we have obtained four placentas from SLE patients -- two preterm and two term. For controls we have obtained five normal, three preterm and two term placentas.

Part of the placentas were fixed for histologic and electron microscopic studies, part were used for establishment of tissue cultures and the remainder frozen for future studies.

For positive control murine placentas from high leukemic strain AKR and low leukemic strain NIH Swiss were used.

In the preterm SLE placenta (so far the only SLE placenta examined by electron microscopy) C-type virus buds and particles were found in the lateral intercellular spaces of cytotrophoblasts. The virus particles are present in various stages of budding. The complete particles are 90-100 ng in diameter and have the typical appearance of C-type virus as seen in AKR or NIH Swiss mice. The relative frequency of particles and buds per area of EM thin section are given below:

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Human SLE placenta	$5.5 \times 10^2/mm^2$
Human Control placenta	$\leq 1/mm^2$
Murine NIH Swiss placenta	$1 \times 10^2/mm^2$
Murine AKR/J placenta	$20 \times 10^2/mm^2$

C. Virological studies. Co-cultivations of SLE trophoblasts with human embryonic fibroblasts, human lymphoblasts, primate fibroblasts, canine embryonic fibroblasts and rat fibroblasts are in progress.

Supernatant fluids from these cultures will be assayed for the presence of an RNA dependent DNA polymerase in association with an endogenous template. DNA probes will be made from the RDDP positive supernatants. They will be hybridized to RNA extracted from murine, feline and primate C-type viruses, as well as to the endogenous RNA that served as template for the synthesis of the DNA probe. If these hybridizations show that the DNA sequences are specific to the RNA on which they were synthesized and do not hybridize to viral RNA from other mammalian C-type viruses, their relationship to human DNA sequences and their evolutionary relationship to other primate DNA will be studied.

A quantitative infectivity assay system similar to the XC test will be developed. We have acquired the personnel and methodology to characterize virus isolates and study virion proteins by biochemical and immunological means.

We have excellent capabilities in (1) production of large amounts of cells and virus, (2) iodination of cell surfaces and virion proteins and (3) determination of their group, type and species specificity by radioimmunoassay.

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11. Publications or papers in press resulting from this work

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12. Summary Progress Report

In the past year we have made considerable progress toward all of the objectives listed in our proposal last year. The report of our activities which follows is listed under the following headings.

1. Effects of neonatal infection of mice and rats with SLV.
2. Immunization of NZB x W female mice with formalinized SLV vaccine.
3. Isolation and characterization of an oncornavirus surface glycoprotein.
4. Taxonomy of Scripps leukemia virus (SLV).
5. Somatotrophic variation of the molecular properties of oncornaviruses.
6. Lack of viral expression in spontaneous tumors of NZB x W F_1 and NZB mice.
7. Biophysical properties of plasma membrane associated DNA.
8. Immunoelectron microscopic study of the structure of nucleic acids.
9. Quantitation and isolation of plasma membrane associated immunoglobulin.
10. Fusion of lymphocytes by feline leukemia virus.
11. Search for C-type viruses in humans.

1. Effects of Neonatal Infection with SLV

In order to determine the oncogenic and autoimmunogenic potential of SLV, the oncornavirus isolated from NZB lymphoblasts (3), we have inoculated SLV into neonatal mice of 16 strains including NZB, NZW and NZB x W. The strains were selected to include both B and N types and a variety of H-2 types so that the influence of these genetic factors in the propagation of virus and causation of disease might be observed.

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